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Bains, B., Greene, M. K., McGirr, L., Dorman, J., Farrow, S., & Scott, C. (2016). Encapsulation of the p38 MAPK inhibitor GSK 678361A in nanoparticles for inflammatory-based disease states. *Journal of Interdisciplinary Nanomedicine*, 1(3), 85-92. <https://doi.org/10.1002/jin2.9>

Published in:
Journal of Interdisciplinary Nanomedicine

Document Version:
Publisher's PDF, also known as Version of record

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ORIGINAL ARTICLE

Encapsulation of the p38 MAPK inhibitor GSK 678361A in nanoparticles for inflammatory-based disease states

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Keywords

inflammation, macrophage, nanoparticle, p38 MAPK inhibitor.

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FUNDING INFORMATION

Royal Society Industrial Fellowship

Received: 20 October 2015;

Revised: 11 March 2016;

Accepted: 31 March 2016

Journal of Interdisciplinary
Nanomedicine,

2016; 1(3), doi: 10.1002/jin2.9

Abstract

Inhibitors of p38 mitogen-activated protein kinase (MAPK) are currently being pursued as therapeutics in inflammatory conditions, but many candidates have demonstrated limited efficacy or toxicity issues to date. Nanoformulation of p38 MAPK inhibitors may overcome these challenges, by enabling controlled release and targeted delivery. Thus, the aim of this study was to develop a nanoformulation of the p38 MAPK inhibitor GSK 678361A and subsequently validate its anti-inflammatory efficacy in vitro, versus the drug in its free format. Poly(lactic-co-glycolic acid) nanoparticles encapsulating GSK 678361A were prepared via a salting-out method and characterised by photon correlation spectroscopy, scanning electron microscopy and high-performance liquid chromatography. The anti-inflammatory effect of both free and nanoformulated GSK 678361A was evaluated in cultures of lipopolysaccharide-stimulated macrophages, with subsequent enzyme-linked immunosorbent assay analysis of TNF- α and IL-6 providing readouts of efficacy. A controlled release nanoformulation of GSK 678361A was successfully developed, with physicochemical characterisation revealing an average particle diameter of 115.5 ± 3.5 nm and polydispersity index of 0.13 ± 0.03 , indicative of a homogeneous size distribution. GSK 678361A loading was quantified at 10.1 ± 0.4 μ g per mg of poly(lactic-co-glycolic acid), equating to an entrapment efficiency of approximately 50%. When tested in cultures of lipopolysaccharide-stimulated macrophages, GSK 678361A nanoparticles inhibited the production of pro-inflammatory cytokines to an extent that was largely comparable with the free drug, although superior efficacy of the nanoformulation was observed at selected doses. These studies indicate that GSK 678361A may be successfully nanoformulated without loss of drug activity, warranting further evaluation in models of inflammation in vivo. © 2016 The Authors. Journal of Interdisciplinary Nanomedicine published by John Wiley & Sons Ltd and the British Society for Nanomedicine

Introduction

p38 Mitogen-activated protein kinase (MAPK) is a serine-threonine kinase that forms part of the mammalian MAPK family, alongside other members such as extracellular signal-regulated kinase and c-Jun NH₂-terminal kinase (Kim and Choi, 2010). It exists in several isoforms, namely, p38- α , - β , - γ and - δ , which are activated in response to environmental stresses or inflammatory cytokines (Cuenda and Rousseau, 2007). Upon activation, p38 MAPK mediates a diverse array of functions, including the regulation of cell differentiation, proliferation and apoptosis (Coulthard *et al.*, 2009). Moreover, it also performs a fundamental role in initiating pro-inflammatory cytokine production and has been shown to modulate cytokine expression at both transcriptional and translational levels (Cargnello and Roux, 2011). As such, p38 MAPK inhibitors are currently being pursued as potential treatment candidates in conditions underpinned by inflammation.

The therapeutic potential of p38 MAPK inhibitors has been examined in several models of inflammatory disease, including rheumatoid arthritis, atherosclerosis, Alzheimer's disease, psoriasis, asthma and chronic obstructive pulmonary disease (Underwood *et al.*, 2000; Nishikawa *et al.*, 2003; Munoz *et al.*, 2007; Medicherla *et al.*, 2008; Medicherla *et al.*, 2010; Seeger *et al.*, 2010). However, progression of these compounds to clinical approval has been limited because of factors such as poor efficacy or adverse side-effects (Cohen *et al.*, 2009; Damjanov *et al.*, 2009; Genovese *et al.*, 2011).

To overcome such limitations, p38 MAPK inhibitors may be integrated within targeted drug delivery systems such as nanoparticles. This strategy affords numerous advantages, including enhanced bioavailability and a reduction in toxicity arising from off-target effects. Moreover, nanoformulation enables sustained release of the payload with a subsequent reduction in dosing schedules and enhanced patient compliance (Gelperina *et al.*, 2005; Parveen *et al.*, 2012).

In this study, we aimed to develop a polymeric nanoparticle platform encapsulating the p38 MAPK inhibitor GSK 678361A (Triantaphyllopoulos *et al.*, 2010), with controlled release properties. We examined the ability of this nanoparticle to inhibit pro-inflammatory cytokine production by murine macrophages in both a time-dependent and dose-dependent manner.

Materials and Methods

Dimethyl sulfoxide (DMSO), acetone, dichloromethane, poly (vinyl alcohol) (PVA), *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), magnesium chloride

hexahydrate, low endotoxin foetal bovine serum and low endotoxin phosphate buffered saline (PBS) were purchased from Sigma Aldrich (UK). Poly(lactic-co-glycolic acid) (PLGA RG502H), with a molecular weight range of 7-17 kDa, was purchased from Evonik Industries (Germany). Slide-A-Lyzer® mini dialysis units 7K MWCO were purchased from Thermo Fisher Scientific Inc (UK). C57BL/6 mice were purchased from Harlan Laboratories (UK). Dulbecco's modified Eagle's medium, penicillin and streptomycin were purchased from Gibco, Life Technologies (UK). *Escherichia coli* R515 lipopolysaccharide (LPS) was purchased from Enzo life sciences (UK). Murine TNF- α and IL-6 DuoSet enzyme-linked immunosorbent assays (ELISAs) were purchased from R&D systems (UK). GSK 678361A was a generous gift from GlaxoSmithKline (UK).

Nanoparticle preparation

GSK 678361A-loaded nanoparticles (GSK 678361A NP) were prepared using an emulsion evaporation salting-out method adapted from McCarron *et al.* (2006). PLGA RG502H (20 mg) was dissolved in 500 μ L acetone and 300 μ L dichloromethane. GSK 678361A dissolved in DMSO (10 mg/mL) was pipetted (40 μ L) into the PLGA mix. The PLGA-drug organic phase was injected dropwise into the aqueous phase (3 mL) comprising 2.5% (w/v) PVA and 45% (w/v) MgCl₂ in CAPS buffer (20 mM, pH 9.0). An emulsion was formed using pulsatory sonication (50 amplitude) on ice for 90 sec. Additional aqueous phase (5 mL) (2.5% w/v PVA in CAPS buffer 20 mM, pH 9.0) was gradually added to the emulsion, under continual stirring, to initiate selective acetone diffusion. Stirring was continued overnight to ensure evaporation of the organic phase. Nanoparticles were purified in CAPS buffer (pH 9) using three wash-centrifugation (60,000 rcf, 20 min, 4°C) cycles.

GSK 678361A entrapment efficiency

GSK 678361A entrapment efficiency was determined using high-performance liquid chromatography (HPLC). The purified NP pellet was dissolved in DMSO and acetonitrile (ACN) (1:3) and measured at 254 nm wavelength using a validated HPLC method. HPLC comprised a Varian Prostar Spectra system with a C-18 HPLC column (50 \times 4.6 mm diameter, 5 μ m pore size) (Phenomenex, UK). The mobile phase (flow rate of 1 mL/min) consisted of solvent A (H₂O) and solvent B (ACN + 0.05% TFA) with elution over a linear gradient of solvent B 10-100% over 5 min. Results were compared to a standard calibration curve of GSK 678361A solution

in DMSO:ACN (1:3). Entrapment efficiency was calculated as follows:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Mass of drug determined (\mu g)}}{\text{Mass of drug added (\mu g)}} \times 100$$

Nanoparticle characterisation

Nanoparticle size and polydispersity index (PDI) were measured in PBS using photon correlation spectroscopy (Zetasizer Nano ZS, Malvern instruments, UK). Measurements were carried out at room temperature. Each sizing determination was carried out in triplicate, and an average particle size was expressed as the mean diameter (Z_{ave}). Routine size measurements and surface morphology were confirmed by scanning electron microscopy (SEM). Nanoparticles were mounted onto copper tape covering an aluminium stub, sputter coated with gold and visualised by SEM (Jeol 6500 field emission gun, Japan).

Evaluation of GSK 678361A release from poly (lactic-co-glycolic acid) nanoparticles

Release profiles of GSK 678361A were determined using a dialysis-based method. A mass of nanoparticles equivalent to approximately 150 μ g GSK 678361A was dispersed in 2 mL growth media (Dulbecco's modified Eagle's medium supplemented with 10% low endotoxin FCS, 1% penicillin and streptomycin). The nanoparticle solution was injected into a pre-wetted Slide-A-Lyzer® dialysis cassette with a 7K molecular weight cut-off (Pierce Biotechnology, USA) using a 21 gauge bevelled needle and immersed into a reservoir of 28 mL pre-warmed growth media. The release study was performed at 37°C under conditions of continuous stirring. Samples (200 μ L) were removed from the reservoir at defined time points. GSK 678361A concentration was measured using a validated HPLC protocol as described previously and compared with a calibration curve for standard GSK 678361A solutions prepared under similar conditions.

Cell preparation and culture

Bone marrow-derived macrophages (BMDM) were isolated from the femur and tibia of C57BL/6 female mice. The femur and tibia were removed from the female donors, and marrow was flushed out using a 23 gauge needle and syringe containing growth media. The bone marrow was pelleted at 650 rcf for 8 min at 4°C, and lysis buffer was used to lyse red blood cells. Cells were differentiated into macrophages by culturing them in the presence of growth media containing

20% L929 supernatant as a source of macrophage-colony stimulating factor. Culture medium was changed on day three to remove non-adherent cells. After seven days, cells were counted and seeded into 96 well plates at a density of 5×10^4 cells/well and allowed to rest overnight in non-L929 supplemented growth media.

Effect of GSK 678361A on lipopolysaccharide-stimulated cytokine production

The efficacy of GSK 678361A in either the free or nano-encapsulated form was assessed using murine BMDM. Cells were stimulated with 100 ng/mL *E. coli* R515 LPS and left to incubate for 1 h prior to further treatment with 2 μ M free GSK 678361A or 2 μ M GSK 678361A NP reconstituted in low endotoxin PBS. Cells were incubated for a further 5 h, after which supernatants were collected and analysed by ELISA.

GSK 678361A dose response

Bone marrow-derived macrophages (BMDM) were pre-treated with LPS as described previously and then further treated with increasing concentrations (ranging from 10^{-4} to 10 μ M/225 μ L) of GSK 678361A suspended in DMSO or GSK 678361A NP reconstituted in low endotoxin PBS. Cells were incubated for a further 5 h, after which supernatants were collected and analysed by ELISA. Cytokine concentrations were compared to those of cells treated with the respective positive controls, DMSO or Blank NP, as a means of calculating therapeutic percentage inhibition of cytokine levels.

Determination of GSK 678361A effect over time

Bone marrow-derived macrophages (BMDM) were treated with 2 μ M free GSK 678361A or 2 μ M GSK 678361A NP reconstituted in low endotoxin PBS at 1 h post-LPS treatment as described previously. Supernatants were collected at pre-determined time points and analysed by ELISA.

Statistical analysis

Statistical significance between data groups was determined using Student's *t*-test or one way analysis of variance (ANOVA) with Tukey post-hoc test. Where statistical significance was achieved, it was represented by asterisks in the appropriate figures and defined as: ****P* < 0.001, ***P* < 0.01, **P* < 0.05. All analyses were performed using GraphPad Prism software (GraphPad software, USA).

Results

Nanoparticle characterisation

Nanoparticle size analysis was performed via photon correlation spectroscopy, revealing an average diameter of 115.5 ± 3.5 nm for GSK 678361A NP (Fig. 1A). The size distribution of the GSK 678361A NP formulation was relatively monodisperse, as indicated by a low PDI value of 0.13 ± 0.03 (Fig. 1A). Using validated HPLC methods, the entrapment efficiency of GSK 678361A within PLGA nanoparticles was measured at $50.6 \pm 2.2\%$, equating to 10.1 ± 0.4 μ g drug per mg of polymer (Fig. 1A). SEM analysis also confirmed the presence of a homogeneous population of nanoparticles, with similar diameters to those obtained via photon correlation spectroscopy (Fig. 1B). Finally, the drug release from these particles was examined at 37°C under continuous stirring conditions, demonstrating a typical biphasic release kinetic, with an initial burst release of $75.7 \pm 8.4\%$ of the drug during the first 24 h, followed by a slower sustained release lasting until the 144 h time point (Fig. 2).

Effect of GSK 678361A on lipopolysaccharide-stimulated cytokine production

Following optimisation of the GSK 678361A NP formulation, functional assays were performed to assess the anti-inflammatory efficacy of both GSK 678361A NP and free GSK 678361A in LPS-stimulated BMDM cultures. As anticipated, LPS stimulation significantly increased TNF- α and IL-6 levels to 427.1 ± 33.6 and 397.7

A

Size (nm)	PDI	Entrapment (μ g / mg PLGA)
115.5 ± 3.5	0.13 ± 0.03	10.1 ± 0.4

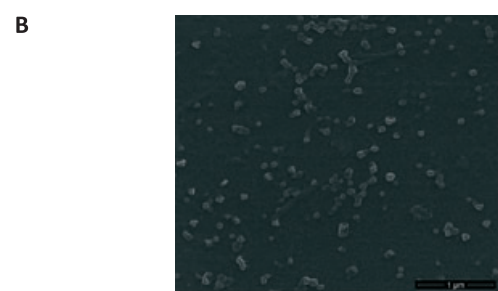


Figure 1. Characterisation of GSK 678361A NP: (A) Size, polydispersity index (PDI) and drug entrapment of GSK 678361A NP. Data expressed as mean \pm SEM. (B) Scanning electron micrograph of GSK 678361A NP. Scale bar = 1 μ m.

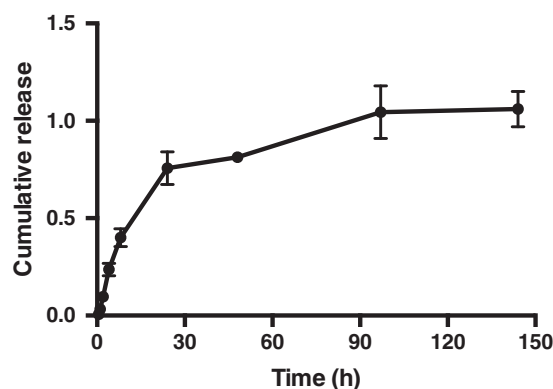


Figure 2. GSK 678361A release profile from PLGA nanoparticles: GSK 678361A NP were resuspended in growth media, injected into a dialysis cassette and incubated at 37°C . Samples were collected at predefined time points, and GSK 678361A concentration was quantified using a validated high-performance liquid chromatography method. Data expressed as mean \pm SEM.

± 4.5 pg/mL, respectively (Fig. 3A and B). Subsequent treatment with 2 μ M GSK 678361A was found to significantly reduce cytokine levels to 113.0 ± 20.4 pg/mL (TNF- α) and 235.9 ± 39.6 pg/mL (IL-6) when used in its free form, and to 132.8 ± 8.4 pg/mL (TNF- α) and 216.2 ± 4.3 pg/mL (IL-6) upon nano-encapsulation. No significant difference in cytokine levels was observed between free and nano-encapsulated GSK 678361A, indicating that both treatments were comparable in efficacy and that drug activity was preserved upon loading into nanoparticles.

GSK 678361A dose response

Having confirmed anti-inflammatory efficacy of free and nano-encapsulated GSK 678361A in BMDM cultures, we next examined the dose-dependency of these effects. Both TNF- α and IL-6 levels were inhibited by GSK 678361A NP in a dose-dependent manner (Fig. 4A and B). Maximal inhibition was observed at a concentration of 10 μ M, where TNF- α and IL-6 levels were significantly reduced by the GSK 678361A NP ($70.9 \pm 3.1\%$ and $61.4 \pm 1.4\%$, respectively), when compared with free GSK 678361A ($50.2 \pm 2.0\%$ and $25.1 \pm 6.0\%$, respectively).

Determination of GSK 678361A effect over time

The inhibitory activity of GSK 678361A in both its free and nano-encapsulated form was next evaluated as a function of time in LPS-stimulated BMDM. At all time points, both free and nano-encapsulated GSK 678361A significantly reduced TNF- α levels when compared with the LPS control (Fig. 5A). The inhibitory effect of GSK 678361A NP on TNF- α production was comparable to

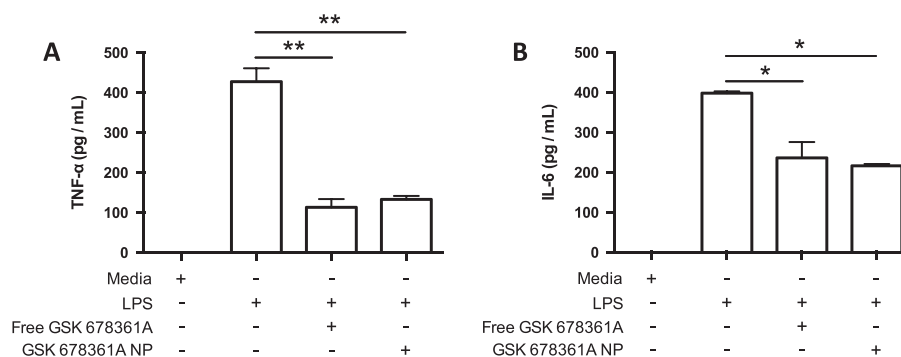


Figure 3. Inhibition of inflammatory cytokine production by GSK 678361A NP: Murine bone marrow-derived macrophages were pre-treated with 100 ng/mL lipopolysaccharide (LPS) for 1 h prior to stimulation with 2 μ M free GSK 678361A or the equivalent amount of GSK 678361A NP. Culture supernatants were collected after 6 h and assayed for both (A) TNF- α and (B) IL-6 by enzyme-linked immunosorbent assay. Statistical significance was assessed by one way ANOVA with Tukey post-hoc test (* $P < 0.05$, ** $P < 0.01$). Data expressed as mean + SEM.

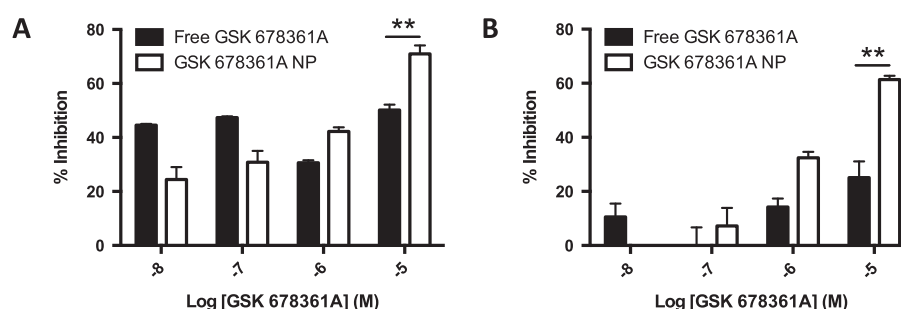


Figure 4. Dose-dependent inhibition of inflammatory cytokine production by GSK 678361A NP: Murine bone marrow-derived macrophages were pre-treated with 100 ng/mL lipopolysaccharide (LPS) for 1 h prior to stimulation with various concentrations of free GSK 678361A or the equivalent amount of GSK 678361A NP. Culture supernatants were collected after 6 h and assayed for both (A) TNF- α and (B) IL-6 by enzyme-linked immunosorbent assay. Statistical significance was assessed by Student's *t*-test (** $P < 0.01$). Data expressed as mean + SEM.

that of the free drug for up to and including the 6 h time point. Similar findings were also noted for IL-6 at 4 and 6 h, although the anti-inflammatory effect of both free and nano-encapsulated GSK 678361A had diminished by 8 h (Fig. 5B).

Discussion

In this current work, we have examined the ability to encapsulate the p38 MAPK inhibitor GSK 678361A, a biphenylamide originally characterised to produce anti-inflammatory effects in a murine collagen-induced arthritis model (Aston *et al.*, 2009; Triantaphyllopoulos *et al.*, 2010). We have demonstrated in this current work that the inhibitor can be successfully formulated into a consistent PLGA nanoparticle formulation, producing anti-inflammatory effects towards LPS-stimulated macrophages.

The p38 MAPKs play central roles in the progression of signaling pathways that promote a number of

pathological conditions such as rheumatoid arthritis and asthma (Schett *et al.*, 2008; Coulthard *et al.*, 2009; Chung, 2011). GSK 678361A was originally developed as an inhibitor of p38- α , although it and the structurally related clinical lead compound, GW856553X (Losmapimod), were both found to selectively inhibit p38- β (Aston *et al.*, 2009), blocking pro-inflammatory cytokine release. Consistent with this earlier work, here, we found that GSK 678361A was able to block release of TNF- α and IL-6 from murine BMDM in both its free and nano-encapsulated form.

Nanoparticle encapsulation represents an area of formulation science aimed at improving the bioavailability and/or delivery of drug cargos to the disease site. Various materials can be employed for the manufacture of such nanoformulations including lipids for the production of liposomes, which remains the most widely used nanomedicine approach applied clinically (Chang and Yeh, 2012). However, other

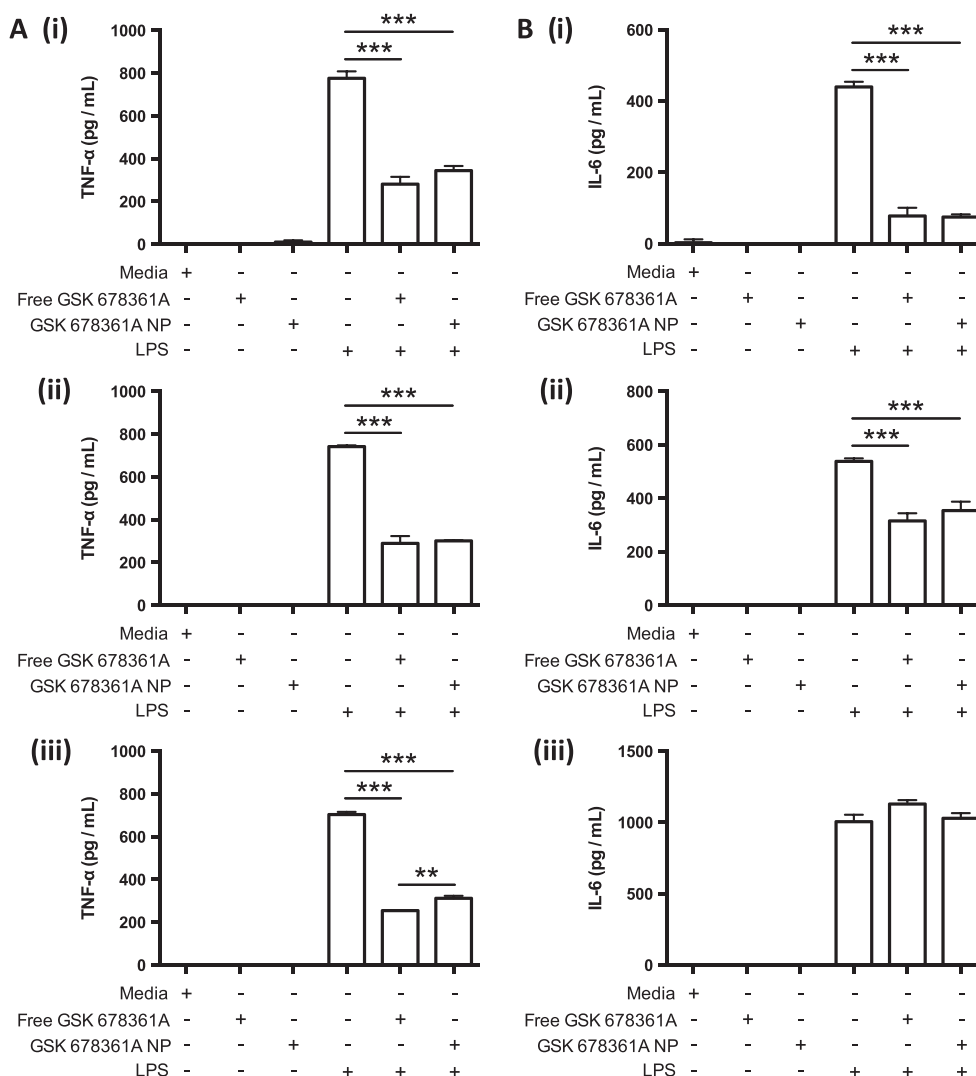


Figure 5. Time-dependent inhibition of inflammatory cytokine production by GSK 678361A NP: (A) Murine bone marrow-derived macrophages were pre-treated with 100 ng/mL lipopolysaccharide (LPS) for 1 h prior to stimulation with 2 μ M free GSK 678361A or the equivalent amount of GSK 678361A NP. Culture supernatants were collected after (i) 4, (ii) 6 and (iii) 8 h and assayed for TNF- α by ELISA. (B) Murine bone marrow-derived macrophages were treated as in (A), and culture supernatants were assayed for IL-6 by enzyme-linked immunosorbent assay. Statistical significance was assessed by one way ANOVA with Tukey post-hoc test (** $P < 0.01$, *** $P < 0.001$). Data expressed as mean \pm SEM.

nanoformulation approaches can employ biodegradable and biocompatible polymer systems, which degrade more slowly than liposomes, providing a sustained or controlled release window (Kumari *et al.*, 2010; Cheng *et al.*, 2015). These include polymer systems such as naturally derived alginate or chitosan nanoparticles, or synthetic PLGA nanoparticles (Dhar *et al.*, 2008; Deacon *et al.*, 2015; Fredman *et al.*, 2015). We have previously worked extensively with PLGA-based nano-systems, demonstrating the ability to enhance the targeting of nanoparticles through attachment of antibodies and other ligands on their surface, generating

therapeutically useful effects (Schmid *et al.*, 2014; Spence *et al.*, 2015).

We have also previously examined polymeric nanoparticle systems to provide sustained release of active pharmaceutical ingredients (APIs), demonstrating enhanced effectiveness of aminoglycoside antibiotics (Abdelghany *et al.*, 2012; Deacon *et al.*, 2015). In this current work, we found that nanoformulation of GSK 678361A provided a typical biphasic controlled release under in vitro conditions, releasing 75% of the drug by 48 h, cumulating in 100% by 4-5 days. Using a range of concentrations, we comparatively examined the ability

of both free and nano-encapsulated drugs to inhibit TNF- α and IL-6 production, showing superior efficacy of nanoformulated GSK 678361A at 10 μ M in particular. These findings indicated that nanoformulation did not abrogate the bioavailability of the drug to the cells. Finally, we examined the persistence of the anti-inflammatory action of GSK 678361A, revealing that both free and nano-encapsulated drug inhibited the production of inflammatory cytokines similarly up to 6 h in vitro. Although TNF- α inhibition was also maintained at 8 h, a loss of efficacy of both free and nano-encapsulated drug was observed for IL-6 at this time point. These findings highlight potential differences in the manner by which p38 MAPK controls individual cytokine production and warrants further investigation.

Beneficial effects of the nano-encapsulation of GSK 678361A were only observed at higher concentrations, although this is not surprising as usefulness of the controlled release effect of the formulation can be difficult to determine in simplistic closed incubation conditions and is in keeping with what we and others have observed previously (Abdelghany *et al.*, 2012; Kolate *et al.*, 2015). However, it is possible that the nanoparticles may elicit further enhanced therapeutic effects using in vivo study models, where the pharmacokinetics of the API can be altered significantly by nano-encapsulation. Furthermore, it is well established that PLGA nanoparticles are relatively hydrophobic and susceptible to opsonisation by macrophages in circulation; given the key role that these cells play in mediating inappropriate inflammation, nanoformulations of GSK 678361A may enhance delivery of the drug to this target cell to provide enhanced effects.

In summary, here, we have developed and characterised a PLGA nanoparticle formulation encapsulating the anti-inflammatory drug GSK 678361A. We have shown that the activity of this API when nanoformulated is equivalent to the free drug and warrants its further evaluation in models underpinned by acute and chronic inflammation.

Acknowledgments

B. K. B., M. K. G., L. M. M. G. and J. D. performed all experiments. B. K. B., M. K. G., S. N. F. and C. J. S. were involved in manuscript preparation. S. N. F. and C. J. S. advised on experimental design. This work was supported in part by a Royal Society Industrial Fellowship awarded to C. J. S. The authors declare no conflict of interest.

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